Cancer stem cells and epithelial-to-mesenchymal transition in lung cancer

Pore, Milind M.

Chapter 8

Summary, Summarizing discussion and Future perspectives

Summary

Lung cancer is a devastating disease with a dismal prognosis [1]. Non-small-cell lung cancer (NSCLC) represents nearly 85% of primary lung cancer malignancies, and is the leading cause of cancer deaths worldwide [2]. Despite the potential benefits of improved diagnostic modalities, approximately 50% of patients with NSCLC present with advanced disease [3]. Small-cell lung cancer (SCLC) is the most aggressive type of all lung cancers and in spite of recent decline in frequency, it still accounts for 12-15% of new lung cancer cases [3]. SCLC is a neuroendocrine tumor that displays a high rate of metastatic dissemination and although initially responsive to therapy, resistance and tumor relapse are inevitable [4].

In most of the cases cancer patients succumb to the disease due to metastases rather than their primary tumors. Currently, the process of metastasis remains poorly understood due to its complexity [5]. It involves several steps such as local invasion of cancer cells into surrounding tissues, transport through microvasculature of lymph and blood system towards distal organs where they colonize at distant tissues and form metastases [6]. Tumor cells within a primary tumor are heterogeneous in nature and hierarchically ordered. The cancer stem cell (CSC) model suggests that within a tumor bulk a subset of tumor cells exist that reside at the apex of hierarchy and possess the capacity of self-renewal, multilineage differentiation and therapy resistance and are thought to play a critical role in the formation of metastasis [7]. Recent evidence also implicates a role for Epithelial to Mesenchymal transition (EMT) in primary tumors for being instrumental in early metastasis [8]. In this process, tumor cells shed their epithelial characteristics and attain more invasive and migratory mesenchymal phenotypes [9]. These tumor cells detach from the primary site and enter the blood circulation, termed as “circulating tumor cells” (CTCs) [10]. CTCs can exhibit properties of both epithelial and mesenchymal phenotypes and their detection has been associated with patient’s survival and treatment prediction in solid tumors including lung cancer [11].

Characterization of CTCs is currently a major focus of translational cancer research and expression of several CSC and EMT markers has been demonstrated in CTCs [12]. Induction of EMT also has been suggested to enhance CSC properties in breast and lung cancer [13,14]. Thus a better understanding of the correlation between EMT, CTCs and CSCs may yield vital clues for developing better diagnostic markers and novel therapeutic strategies that will lead to better management of lung cancer.

In this thesis we focused on the role of CSC, EMT and CTC in lung cancer progression, metastasis, and therapy resistance.

Summary of thesis:

In **chapter 1**, a general introduction is given on lung cancer, its subtypes and pathophysiology. Moreover, an introduction on CSC, EMT and CTCs is provided together with the scope and aim of this thesis and outline of the subsequent chapters.

In **chapter 2** the possible involvement of EMT and CSCs in resistance to therapy and metastasis was investigated in a unique longitudinal SCLC model consisting of three cell lines previously derived from one SCLC patient. Cell lines were derived from different biopsies obtained prior to any treatment (GLC14), after treatment, at first recurrence (GLC16) and at second recurrence with a more clinically resistant stage of disease (GLC19) respectively. These cell lines did not show significant differences in chemosensitivity for different chemotherapeutics previously [15,16]. We hypothesized that EMT and enrichment of CSC characteristics would occur during disease progression. All three cell lines expressed the epithelial markers EpCAM and E-cadherin whereas none of the cell lines displayed expression of the mesenchymal markers Vimentin and Fibronectin. The GLC14 cell line, with cells derived from a metastatic lymph node biopsy instead of the primary tumor, showed the strongest invasive capacity and spheroid forming potential of all tested cell lines *in vitro*. Expression of the known CSC markers (CD44 and SOX2) increased from GLC14 to GLC19 cells. Interestingly, GLC14 cells displayed the highest spheroid forming potential amongst the three cell lines, indicating that this lymph node derived cell line has different properties than GLC16 and GLC19 cells. GLC19 showed an increased spheroid forming potential compared to GLC16 suggestive of enhanced CSC properties during disease progression. Moreover, CD44+ sorted cells showed enhanced SOX2 expression and increased spheroid forming potential compared to CD44- sorted cells in all three cell lines upon growth in serum-free neurobasal media (NBM). To conclude, we could not demonstrate differences in EMT marker expression in this cell line panel reflecting disease progression, however observed an increase in several CSC properties. Taken together, our results suggest that SCLC cells acquire enhanced CSC properties during disease progression and that more distant lesions differ in invasive capacity compared to the primary tumor.

In **chapter 3** we used the well-known TGF-β inducible A549-NSCLC EMT model to study the possible effects of EMT on chemosensitivity, migration potential, invasive capacity and CSC properties. EMT was confirmed in TGF-β treated A549 cells with cells showing fibroblast like cell morphology, loss of the epithelial markers EpCAM and E-cadherin and upregulation of the mesenchymal markers Fibronectin and Vimentin. Mesenchymal cells displayed somewhat enhanced chemoresistance towards cisplatin and an increased migration and invasive potential compared to epithelial A549 cells. Spheroid forming assays of A549 cells in the presence of TGF-β showed higher spheroid forming potential in TGF-β treated cells. Moreover these cells also showed elevated expression of the CSC markers OCT4 and SOX2. We used stably transfected luciferase labeled A549 (A549-luc) cell line to set up an orthotopic mouse model to compare metastatic spread of epithelial and mesenchymal cells. Bioluminescent imaging (BLI) and pathological examination in preliminary experiments confirmed massive tumor growth at the primary site, in the adjacent lung and metastatic lesions in the liver of all animals. Also in both groups distant metastases were detected at kidneys, brain and adrenal glands. Attempts to detect CTCs by BLI in blood samples of the mice were not successful possibly due to low sensitivity of the detection system. In conclusion, mesenchymal A549 cells displayed enhanced migration, invasion, chemoresistance and increased CSC properties compared to epithelial counterparts. The pilot experiment using the lung orthotopic mouse model showed tumor growth and metastatic spread in both epithelial and mesenchymal cells. Further optimization is required to monitor possible differences in metastatic spread between these phenotypes.

In **chapter 4** we described a multicenter clinical study with 59 SCLC patients. The aim of this study was to investigate the prognostic and predictive value of CTCs in patients with SCLC. For this purpose CTC levels were detected at baseline, after 1 cycle and after 4 cycles of chemotherapy using the Veridex CellSearch platform. At baseline, lower numbers of CTCs were observed in 21 patients with limited disease (median = 6, range 0–220) compared with 38 patients with extensive disease (median = 63, range 0–14 040). Lack of measurable CTCs (27% of patients) was associated with prolonged survival (HR 3.4; $P \leq 0.001$). CTCs decreased after one cycle of chemotherapy; this decrease was not associated with tumor response after four cycles of chemotherapy. CTC count after the first cycle of chemotherapy was the strongest predictor for overall survival (HR 5.7; 95% CI 1.7–18.9; $P = 0.004$). We conclude that CTC levels are useful prognostic marker in SCLC; patients with lower initial CTC count live longer than those with high CTC count. Furthermore, CTC

count after 1st cycle of chemotherapy is a strong predictor for response to chemotherapy and survival.

In **chapter 5** we used the biopsy material from patients that enrolled in the clinical study described in chapter 4 in order to examine a possible correlation between CSC or EMT marker expression, CTC levels and prognosis. We hypothesized that high levels of CSC and EMT markers would be associated with high CTC levels and poor prognosis in patients. Biopsies of 38 SCLC patients at diagnosis were used for marker evaluation by immunohistochemistry. Expression of the CSC markers CD44 and SOX2 and EMT markers E-cadherin, EpCAM, c-MET, Vimentin and Cytokeratin 8, 18,19 was evaluated. High expression of c-MET (c-MET^{H}) and low expression level of E-cadherin (E-cad^{L}) showed a trend towards better prognosis (p=0.07 and p=0.09 respectively). When these markers were combined (c-MET^{H}E-cad^{L}) a significant correlation towards better survival was found (p=0.007). No correlation of the tested markers with baseline CTC count was observed. However, there was a trend for c-MET^{H}E-cad^{L} (p=0.09) with low baseline CTC count. CSC markers SOX2 and CD44 were not associated with overall survival in this patient cohort. Our results suggest that SCLC with a mesenchymal-like phenotype (c-MET^{H}E-cad^{L}) is associated with longer survival and showed a trend towards lower baseline CTCs.

In **chapter 6** we investigated the potential of CSC enrichment of esophageal adenocarcinoma (EAC) cells in an *in vitro* three dimensional spheroid cell culture model. The EAC cell line OE19, when cultured as spheroids under serum free conditions (OE19S), showed enhanced CSC properties as determined by spheroid formation and chemoresistance assays *in vitro*. This enrichment for CSC characteristics was not seen in another spheroid-cultured EAC cell line, OE33S, when compared to monolayer OE33 cells. However, following implantation in NOD/SCID mice spheroid-cultured OE19S, but also spheroid cultured OE33S cells displayed enhanced tumor growth compared to monolayer-derived xenografts. mRNA expression of spheroid and monolayer cultures of OE19 and OE33 cells, as well as their xenografts, was determined using an Illumina array. Expression of the stem cell associated genes *KLF2* and *C-FOS* was significantly and at least 2-fold increased in spheroid cultures of both the cell lines compared to their monolayer counterpart. No significant differences in gene expression were seen between OE19S and OE33S derived xenografts models. qRT PCR results were in concordance with the microarray data, except for *KLF2* for the OE33 model. Gene Set Enrichment analysis showed alterations in DNA regulation and cell adhesion

pathways between monolayer and spheroid cultured cells. To conclude, the OE19 spheroid model could be used to study CSC characteristics in EAC and deserves further validation. However, not all EAC spheroid cell line models appear to follow the predictions of the CSC model, and caution should be taken when interpreting results. The genes that we found differentially expressed between monolayer and spheroid-cultured EAC cells warrant further investigation for their possible role in CSC in EAC.

In **chapter 7** we conducted a literature study on current and novel methods of targeting apoptosis pathways in lung cancer as therapeutic strategies. We included approaches aimed at targeting tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, BCL-2 family members and apoptosis-inhibitory proteins (IAPs). Therapeutics that modulate apoptosis are more effective when used in combination with traditional cytotoxic chemotherapy. We also discussed preclinical and clinical trials that were conducted or ongoing and the future perspectives. We propose that it would be beneficial to test the combination of apoptosis targeting agents such as BCL-2 inhibitors with the TRAIL receptor targeting agents or BCL-2 inhibitors with EGFR inhibitors. Novel biomarkers that can predict the responses towards these drugs have yet to be identified.

Summarizing Discussion and Future Perspectives

CSCs and EMT in SCLC

Our study in the longitudinal SCLC model representing disease progression showed no correlation with EMT marker expression, whereas some association with an increase in CSC properties was seen. SCLC cells derived from metastatic biopsies (GLC14) showed different invasive and CSC properties compared to the cells derived from primary endobronchial biopsies possibly indicative of altered properties in metastasized SCLC cells. In earlier studies CSCs have been isolated from SCLC patient's tumor biopsies on the basis of cell surface marker expressions such as CD133, CD44 and by side population (SP) analysis [17]. However, there are no comparative studies as yet between CSC populations isolated from primary and metastatic biopsies, though it is likely that these populations will differ. This seems to be reflected by GLC14, derived from a metastasized lymph node, displaying the highest invasive and spheroid forming potential. Thus, CSC properties may be dependent on the site from which they are derived (i.e. primary tumor vs metastatic site). In line with that, it has been suggested that two different kinds of CSC populations exist namely stationary CSCs and migrating CSCs in colorectal cancer

[18]. Perhaps GLC14 cells are derived from migratory CSCs, which have enhanced invasive potential.

We used several known CSC markers, however, additional CSC markers and functional assays such as ALDH activity and SP analysis could be used to further characterize this longitudinal SCLC cell line model for CSC properties. In addition, the GLC cells and sorted fractions could be tested for tumorigenicity and CSC enrichment by limiting dilution assays in NOD/ SCID mouse. Although several CSC specific markers have been identified in SCLC their role in disease relapse and resistance has not yet been firmly demonstrated. There are conflicting results with candidate CSC markers, their prognostic relevance and their use as novel therapeutic targets. Most of these studies in SCLC rely on cell line models and *in vitro* assays, which likely do not fully represent tumor heterogeneity in the actual tumor. Moreover most of the CSC markers used in lung cancer including SCLC were identified in other types of solid tumors and leukemia and their utility for lung needs to be thoroughly investigated. Future experiments employing primary tumor material and an experimental setup mimicking the natural tumor microenvironment such as orthotopic xenografts or three dimensional organoid models [19] may lead to new insights in identifying lung CSC properties.

We could not demonstrate indigenous occurrence of EMT in this longitudinal SCLC cell line model that was limited to the detection of only a number of EMT markers. Perhaps the biopsies from which the cell lines were derived may not represent the cells that were able to undergo EMT or *in vitro* culturing might have selected only one type of phenotype (epithelial) to dominate over other with time.

EMT and CTCs in SCLC

We found that CTC levels determined by the Veridex CellSearch platform are good prognostic and possible predictive markers in SCLC. Our findings indicate that patients with low levels of CTCs and with limited disease (LD) have a better overall survival compared to patients with high CTCs and with extensive disease (ED). Moreover CTC count after 1st cycle of chemotherapy showed a strong predictive value for response to chemotherapy and survival.

Our results are in line with several clinical studies that correlated high CTC values with worse prognosis. The first large clinical study on CTCs was carried out in breast cancer [20] and subsequently high CTC values were correlated with bad prognosis

in other tumor types such as colorectal [21], prostate [22] and in NSCLC [23]. In line with our study described in chapter 4 [24], Hou et al demonstrated that CTCs in SCLC are prognostic and predictive markers [25]. Moreover they reported the presence of large CTC clusters, termed circulating tumor microemboli (CTM). These CTM did not show the presence of apoptotic cells in contrast to single CTCs, which may implicate that cell-cell interactions in CTM prevent anoikis leading to higher levels of vital CTCs able to metastasize. In many of the mentioned clinical studies, CTC levels were used to predict the treatment efficacy of solid tumors. This could be relevant, implying that if there is no reduction in CTC levels following one course of treatment, patients could be withdrawn from administrating the same therapeutic regime, which would be cost effective and avoid unnecessary side effects to the patients. Interestingly, a recent study by Smerage et al demonstrated that early switching to an alternative therapy based on persistent CTCs did not improve the overall survival in metastatic breast cancer patients. This suggests that there is a need for more effective treatments than the current standard chemotherapy regimen [26].

Veridex CellSearch is currently the only FDA approved CTC detection method. Apart from CellSearch several other methods use EpCAM for capturing the CTCs such as microfluidic devices like the CTC-chip, Herringbone chip, iCHIP (All Massachusetts General hospital center USA), IsoFlux (Fluxion USA), MagSweeper (Stanford University, USA) and GILUPI Nanomedezin (Germany) [27]. However not all CTCs express epithelial markers; cells that have undergone EMT or partial EMT lose their epithelial markers and attain more mesenchymal markers and will escape detection by EpCAM-based CTC capturing methods. Thus other technologies that are independent of cell surface marker expressions and which are based on physical properties such as size and density are gaining more attention. This includes isolation by size of tumor cells (ISET), Dean Flow Fractionation, isolation by density (OncoQuick) and isolation by membrane capacitance like Dielectropheretic filed- flow fractionation. Other methods are based on functional assays such as EPithelial Immuno SPOT (EPISPOT), density gradient centrifugation followed by growing cells to Chick Chorioallantoic Membrane (CAM) for short-term culture, high-throughput fluorescent scanning that includes red blood cell lysis and density gradient centrifugation and DEPArray which is based on single cell sequencing technology [27]. Although these techniques are efficient, limited clinical evidences are currently available to confirm their advances over FDA approved CellSearch technology.

We also performed an immunohistochemical study for the detection of EMT and CSC markers in the samples from the same SCLC patients that enrolled in the CTC clinical study. We concluded that the patients with a mesenchymal-like phenotype (c-MET^{H}E-cad^{L}) were associated with significantly better prognosis and showed a trend towards low baseline CTC counts. Our results are contradictory with previous evidences regarding the expression of EMT markers and their association with prognosis in SCLC and other solid tumors. Most of the current literature suggests that an epithelial phenotype is associated with better prognosis whereas a mesenchymal phenotype reflects the presence of high migratory and invasive tumor cells, that is associated with worse outcome [28]. Previously, expression of the epithelial marker E-cadherin was associated with a better prognosis in SCLC [29], whereas amplification of the EMT transcription factor ZEB1 was associated with bone metastasis in SCLC patients [30]. In the same study, siRNA knockdown of ZEB1 in a SCLC bone metastasis derived cell line inhibited the migration and invasive potential of these cells as well reduced bone metastasis. Overexpression of c-MET has been associated with worse outcome in SCLC and c-MET inhibition with a small molecular inhibitor PHA-665752 resulted in survival benefit to SCLC patients [31]. However our results are in line with *in vitro* findings in a SCLC cell line H69, where floating aggregates expressing high epithelial characters resulted in enhanced *in vivo* tumorigenicity in subcutaneous mouse model [32]. SCLC shows both neuroendocrine and non- neuroendocrine/ epithelial differentiation suggesting that SCLC tumors may have a common epithelial ancestor [33]. Earlier, presence of both these phenotypes has been demonstrated in SCLC patient blood, moreover neuroendocrine phenotype as measured by Pro-opiomelanocortin (POMC) was correlated with worse prognosis, liver metastasis and epithelial positive CTCs [34]. As mentioned previously, CTC detection by Veridex CellSearch platform will fail to detect EpCAM low/negative mesenchymal CTCs and, in addition, may also miss circulating neuroendocrine SCLC cells that may remain undetected. Recent study showed that microcavity array (MCA) system, a novel CTC isolation method based on cell size could detect CTCs and CTC clusters from SCLC patients that were missed by Veridex technology [35].

Interestingly, a new and contradictory “epithelial-mesenchymal co-operation model” has been suggested recently in SCLC [27]. In mice it was demonstrated that subcutaneous injections of mixed epithelial and neuroendocrine SCLC cells are required for liver metastasis. Neither of the cell types resulted in metastasis if injected alone or separately at opposite flanks [36]. In another study subcutaneous

injections of epithelial and mesenchymal cells of hamster oral keratinocytes resulted in intravasation of both types of tumor cells, however, only epithelial cells resulted in lung metastasis suggesting that mesenchymal cells may facilitate the invasion of both type of tumor cells but only epithelial cells result in distant metastasis [37]. Hou et al found that that CTCs and CTM isolated by ISET from SCLC patients displayed heterogeneous expression of E-cadherin and Vimentin, however, none of them expressed both the markers suggestive of a mixed population of epithelial and mesenchymal circulating SCLC cells [11]. Anyway, regardless of the underlying mechanisms it will be important in future to count CTCs using a marker-independent method such as ISET in a large SCLC patient cohort in order to corroborate the association between EMT markers expression, CTC counts and prognosis in SCLC.

Recently CTC derived explant (CDX) mouse model have been developed that mimic the donor patient's response to therapy. These CDX models also show similarity in genomic analysis with their originating CTCs and can be used to study the resistance mechanism and testing new therapeutics in SCLC [38]. Further research using advanced technologies such as single cell sequencing, next generation sequencing (NGS) and whole genome analysis (WGA) may provide new insights to identify the metastatic initiating cells or therapy resistant phenotypes that may help to design novel therapeutic strategies for better management of lung cancer progression. Finally, CTC capturing with novel biomarkers such as actin-bundling protein plastin 3 which can capture both epithelial and mesenchymal CTCs as well as novel detection methods that allows characterization and their *in vitro* culturing are currently underway [39,40].

EMT in NSCLC

Our data conclude that TGF-β treated A549 (mesenchymal) cells display enhanced chemoresistance, migration and invasion potential as well as CSC properties compared to the untreated A549 (epithelial) cells. Our *in vitro* results are in accordance with previous studies. Earlier, acquired docetaxel resistance in A549 cells displayed mesenchymal features [41] and EMT induction in A549 cells resulted in resistance to gefitinib and erlotinib and enhanced migration and invasion potential [42]. In A549 cells activation of EMT by TGF-β showed transcriptional upregulation of Sonic Hedgehog pathway that was associated with increased migration, invasion, and *in vitro* colony forming potential [43]. Most of the evidences for EMT come from *in vitro* studies and from immunohistochemical staining studies of patient material where expression of EMT markers is monitored and compared with patient's

clinicopathological features. In NSCLC the presence of E-cadherin has been associated with longer overall and disease free survival [44] and expression of Vimentin has been associated with poor disease free survival [45] suggesting that the epithelial phenotype associates with better outcome.

Recently induction of EMT has been associated with enhanced CSC properties in breast cancer [13]. In NSCLC cell line models, TGF-β induced EMT showed elevated expressions of CSC specific markers OCT4, NANOG, SOX2 and CD133 [14]. In another study, the same group demonstrated that CD133 positive as well as side population (SP) A549 cells had enhanced motility [46]. EMT has been also demonstrated in preclinical mouse models. For example, in a breast cancer mouse model EMT occurring in the primary tumor was associated with enhanced intravasation and generation of CTCs where characterization of CTCs displayed high expression of Vimentin [47].

Targeting EMT seems an attractive anti-cancer strategy. There are at least four stages where EMT can be targeted using pharmacological drugs [48]. First, EMT can be inhibited using antagonists of extracellular EMT-inducing stimuli. For example blocking of EGF receptor kinase using small molecular inhibitor AG1478 and TGF-β receptor kinase using SB431542 have been reported to inhibit EMT in endometrial carcinoma and pancreatic cancer cells [49,50] A second approach is the specific targeting of signaling pathways mediating EMT like signal transducer and activator of transcription-3 (STAT3) using inhibitors such as Stattic and S3I-201 [51,52]. Thirdly, direct targeting of a mesenchymal marker such as Vimentin with weithaferin-A that is able to prevent Vimentin assembly and functioning lead to inhibition of cell migration and invasion in lung and breast cancer cells and also inhibited metastasis formation *in vivo* [53,54]. Inhibition of other mesenchymal markers such as N-cadherin using monoclonal antibodies has been reported [55]. A fourth approach is the targeting of Mesenchymal to Epithelial transition (MET) thought to be required for tumor colonization at secondary sites using the FGF receptor isoform FGFR2IIIc as was reported for a bladder carcinoma cell line [56].

Each of the above mentioned strategies have their own advantages and limitations. For example inhibiting mesenchymal markers/ EMT markers may lead to epithelialisation of disseminated tumor cells leading to metastasis, whereas targeting MET may lead to mesenchymalisation and therapy resistance. Inhibiting mesenchymal cells may lead to alternative forms of cell migration like amoeboid

migration which may be the reason for failure of the phase III protease inhibitor clinical trial in breast cancer [57]. Most of times at diagnosis tumor cells have already metastasized and inhibiting EMT is no more an option at that stage, although it may prevent further dissemination. Further research on each of these approaches is required to examine if targeting specific stages of EMT will be beneficial.

CSCs in Esophageal Adenocarcinoma (EAC)

In this thesis we described three-dimensional spheroid culturing of the EAC cell line OE19 in serum-deprived medium displaying enhanced CSC characteristics. This model may be useful to further explore the CSC model in EAC, including limited dilution assays *in vivo*. We found upregulation of *C-FOS* and *KLF2* in the spheroid model and RNA interference-based knockdown of these genes and subsequent testing the effect on spheroid formation *in vitro* and tumor forming ability in mice, will establish their possible involvement in cancer stemness. However, we observed that spheroid growth does not always enrich for CSC properties, because spheroids grown from the OE33 cell line did not show significant enhancement of such characteristics *in vitro*. Perhaps medium-induced reprogramming will not be possible in all cancer cells, and/or cells may already have a CSC phenotype that cannot be enhanced further. Therefore caution should be taken when using spheroid models as enriched CSC models and each spheroid model should be characterized independently. A better understanding of the CSC model in esophageal oncogenesis and disease progression may provide new leads for targeted therapy. While several markers have been reported to associate with esophageal CSCs, a definitive characterization of CSCs in EAC has not yet been clearly established. CD44, CD90 and p75NTR have been suggested to enrich for CSC-like cells in esophageal cancer [58–60]. A broad panel of possible CSC markers, including CD44 and CD24, were tested using primary patient material however neither of these markers enriched for cells with enhanced tumorigenicity *in vivo* [61]. Besides the use of markers, functional assays can also enrich for CSCs. The side-population assay, based on Hoechst exclusion assay, enriched for EAC cells with CSC-characteristics demonstrated by enhanced tumorigenicity *in vivo* [62].

To further explore the CSC model in EAC, the use of primary tumor cells derived from fresh patient material might provide a better model system. An alternative approach to study CSCs in EAC could involve mouse models that mimic EAC development. In a transgenic Barrett esophagus mouse model overexpressing

interleukin-1-β (IL-1-β) and crossed with a Lgr5- Cre-ERT/Rosa-LacZ reporter mouse, Lgr5 positive cells were shown to migrate from the cardia and were found in the metaplastic Barrett's epithelium [63]. The combination of lineage tracing of labelled stem or progenitor cells in the normal squamous epithelium together with an inducer of malignant progression (such as IL-1-β overexpression), could be another approach to identify potential mechanisms in CSCs driving EAC development.

Conclusions

EMT, CSC and CTCs are thought to play an important role in cancer progression and metastatic spread of disease. However, the relationship between these processes remains poorly understood. In this thesis we have addressed the association between these processes and their role in disease progression and prognosis. We provided *in vitro* support for an increase in CSC but not EMT features in SCLC during disease progression, and found that induction of EMT in A549 cells enhanced CSC properties. CTC counts were found to reflect disease status in SCLC patients and to be prognostic predictors for response to chemotherapy. However, we could not link a mesenchymal status with higher CTC levels in part of the same cohort of patients. Therefore, these findings do not justify the drawing of firm conclusions on the possible connection between CSC, EMT and CTCs. *In vitro* the mechanisms driving EMT and CSCs need to be studied in more detail, which will also reveal the overlaps in pathways. Furthermore, many issues are still not clear, for example, how EMT guides dissemination of tumor cells from primary site to blood, how CTCs are protected from anoikis, and if CTCs have CSC properties that are implicated in metastatic spread of disease. *In vivo* imaging techniques allowing the monitoring of the actual behavior of specific tumor cells in mice will be important in this respect. Ultimately, research on mechanisms controlling EMT and CSC may provide new therapeutic targets. Genomic and proteomic characterizing of CTCs at single cell level will yield vital clues for identifying the mechanisms behind drug resistance, metastasis initiating cells and tumor recurrence. Monitoring of CTCs as a non-invasive liquid biopsy could be integrated in cancer treatment for diagnostic purposes, and *in vitro* culturing of CTCs may provide a means to test drug efficacy that could then be translated to the clinic.

References

[1] R. Siegel, J. Ma, Z. Zou, and A. Jemal, “Cancer statistics, 2014.,” *CA. Cancer J. Clin.*, vol. 64, no. 1, pp. 9–29, Jan. 2014.

[2] R. Govindan, N. Page, D. Morgensztern, W. Read, R. Tierney, A. Vlahiotis, E. L. Spitznagel, and J. Piccirillo, “Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the surveillance, epidemiologic, and end results database.,” *J. Clin. Oncol.*, vol. 24, no. 28, pp. 4539–44, Oct. 2006.

[3] A. Jemal, R. Siegel, J. Xu, and E. Ward, “Cancer statistics, 2010.,” *CA. Cancer J. Clin.*, vol. 60, no. 5, pp. 277–300, Jan. 2010.

[4] I. U. Amarasena, J. A. E. Walters, R. Wood-Baker, and K. Fong, “Platinum versus non-platinum chemotherapy regimens for small cell lung cancer.,” *Cochrane database Syst. Rev.*, no. 4, p. CD006849, Jan. 2008.

[5] C. L. Chaffer and R. A. Weinberg, “A perspective on cancer cell metastasis.,” *Science*, vol. 331, no. 6024, pp. 1559–64, Mar. 2011.

[6] D. X. Nguyen, P. D. Bos, and J. Massagué, “Metastasis: from dissemination to organ-specific colonization.,” *Nat. Rev. Cancer*, vol. 9, no. 4, pp. 274–84, Apr. 2009.

[7] B. J. Morrison, J. C. Morris, and J. C. Steel, “Lung cancer-initiating cells: a novel target for cancer therapy.,” *Target. Oncol.*, vol. 8, no. 3, pp. 159–72, Sep. 2013.

[8] H. Liu, X. Zhang, J. Li, B. Sun, H. Qian, and Z. Yin, “The biological and clinical importance of epithelial-mesenchymal transition in circulating tumor cells.,” *J. Cancer Res. Clin. Oncol.*, Jun. 2014.

[9] B. De Craene and G. Berx, “Regulatory networks defining EMT during cancer initiation and progression.,” *Nat. Rev. Cancer*, vol. 13, no. 2, pp. 97–110, Feb. 2013.

[10] J. D. O’Flaherty, S. Gray, D. Richard, D. Fennell, J. J. O’Leary, F. H. Blackhall, and K. J. O’Byrne, “Circulating tumour cells, their role in metastasis and their clinical utility in lung cancer.,” *Lung Cancer*, vol. 76, no. 1, pp. 19–25, Apr. 2012.

[11] J.-M. Hou, M. Krebs, T. Ward, R. Sloane, L. Priest, A. Hughes, G. Clack, M. Ranson, F. Blackhall, and C. Dive, “Circulating tumor cells as a window on metastasis biology in lung cancer.,” *Am. J. Pathol.*, vol. 178, no. 3, pp. 989–96, Mar. 2011.

[12] N. Krawczyk, F. Meier-Stiegen, M. Banys, H. Neubauer, E. Ruckhaeberle, and T. Fehm, “Expression of stem cell and epithelial-mesenchymal transition markers in circulating tumor cells of breast cancer patients.,” *Biomed Res. Int.*, vol. 2014, p. 415721, Jan. 2014.

[13] S. A. Mani, W. Guo, M.-J. Liao, E. N. Eaton, A. Ayyanan, A. Y. Zhou, M. Brooks, F. Reinhard, C. C. Zhang, M. Shipitsin, L. L. Campbell, K. Polyak, C. Brisken, J. Yang, and R. A. Weinberg, "The epithelial-mesenchymal transition generates cells with properties of stem cells.," *Cell*, vol. 133, no. 4, pp. 704–15, May 2008.

[14] G. Pirozzi, V. Tirino, R. Camerlingo, R. Franco, A. La Rocca, E. Liguori, N. Martucci, F. Paino, N. Normanno, and G. Rocco, "Epithelial to mesenchymal transition by TGFβ-1 induction increases stemness characteristics in primary non small cell lung cancer cell line.," *PLoS One*, vol. 6, no. 6, p. e21548, Jan. 2011.

[15] H. H. Berendsen, L. de Leij, E. G. de Vries, G. Mesander, N. H. Mulder, B. de Jong, C. H. Buys, P. E. Postmus, S. Poppema, and H. J. Sluiter, "Characterization of three small cell lung cancer cell lines established from one patient during longitudinal follow-up.," *Cancer Res.*, vol. 48, no. 23, pp. 6891–9, Dec. 1988.

[16] E. G. de Vries, C. Meijer, H. Timmer-Bosscha, H. H. Berendsen, L. de Leij, R. J. Scheper, and N. H. Mulder, "Resistance mechanisms in three human small cell lung cancer cell lines established from one patient during clinical follow-up.," *Cancer Res.*, vol. 49, no. 15, pp. 4175–8, Aug. 1989.

[17] T. Sourisseau, K. A. Hassan, I. Wistuba, F. Penault-Llorca, J. Adam, E. Deutsch, and J.-C. Soria, "Lung cancer stem cell: fancy conceptual model of tumor biology or cornerstone of a forthcoming therapeutic breakthrough?," *J. Thorac. Oncol.*, vol. 9, no. 1, pp. 7–17, Jan. 2014.

[18] T. Brabletz, A. Jung, S. Spaderna, F. Hlubek, and T. Kirchner, "Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression.," *Nat. Rev. Cancer*, vol. 5, no. 9, pp. 744–9, Sep. 2005.

[19] R. E. Hynds and A. Giangreco, "Concise review: the relevance of human stem cell-derived organoid models for epithelial translational medicine.," *Stem Cells*, vol. 31, no. 3, pp. 417–22, Mar. 2013.

[20] M. Cristofanilli, G. T. Budd, M. J. Ellis, A. Stopeck, J. Matera, M. C. Miller, J. M. Reuben, G. V Doyle, W. J. Allard, L. W. M. M. Terstappen, and D. F. Hayes, "Circulating tumor cells, disease progression, and survival in metastatic breast cancer.," *N. Engl. J. Med.*, vol. 351, no. 8, pp. 781–91, Aug. 2004.

[21] S. J. Cohen, C. J. A. Punt, N. Iannotti, B. H. Saidman, K. D. Sabbath, N. Y. Gabrail, J. Picus, M. Morse, E. Mitchell, M. C. Miller, G. V Doyle, H. Tissing, L. W. M. M. Terstappen, and N. J. Meropol, "Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer.," *J. Clin. Oncol.*, vol. 26, no. 19, pp. 3213–21, Jul. 2008.

[22] H. I. Scher, X. Jia, J. S. de Bono, M. Fleisher, K. J. Pienta, D. Raghavan, and G. Heller, "Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data.," *Lancet. Oncol.*, vol. 10, no. 3, pp. 233–9, Mar. 2009.

[23] M. G. Krebs, R. Sloane, L. Priest, L. Lancashire, J.-M. Hou, A. Greystoke, T. H. Ward, R. Ferraldeschi, A. Hughes, G. Clack, M. Ranson, C. Dive, and F. H. Blackhall, "Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer.," *J. Clin. Oncol.*, vol. 29, no. 12, pp. 1556–63, Apr. 2011.

[24] T. J. N. Hiltermann, M. M. Pore, A. van den Berg, W. Timens, H. M. Boezen, J. J. W. Liesker, J. H. Schouwink, W. J. A. Wijnands, G. S. M. A. Kerner, F. A. E. Kruyt, H. Tissing, A. G. J. Tibbe, L. W. M. M. Terstappen, and H. J. M. Groen, "Circulating tumor cells in small-cell lung cancer: a predictive and prognostic factor.," *Ann. Oncol.*, vol. 23, no. 11, pp. 2937–42, Nov. 2012.

[25] J.-M. Hou, M. G. Krebs, L. Lancashire, R. Sloane, A. Backen, R. K. Swain, L. J. C. Priest, A. Greystoke, C. Zhou, K. Morris, T. Ward, F. H. Blackhall, and C. Dive, "Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer.," *J. Clin. Oncol.*, vol. 30, no. 5, pp. 525–32, Feb. 2012.

[26] J. B. Smerage, W. E. Barlow, G. N. Hortobagyi, E. P. Winer, B. Leyland-Jones, G. Srkalovic, S. Tejwani, A. F. Schott, M. A. O'Rourke, D. L. Lew, G. V Doyle, J. R. Gralow, R. B. Livingston, and D. F. Hayes, "Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500.," *J. Clin. Oncol.*, vol. 32, no. 31, pp. 3483–9, Nov. 2014.

[27] M. G. Krebs, R. L. Metcalf, L. Carter, G. Brady, F. H. Blackhall, and C. Dive, "Molecular analysis of circulating tumour cells-biology and biomarkers.," *Nat. Rev. Clin. Oncol.*, vol. 11, no. 3, pp. 129–44, Mar. 2014.

[28] M. Iwatsuki, K. Mimori, T. Yokobori, H. Ishi, T. Beppu, S. Nakamori, H. Baba, and M. Mori, "Epithelial-mesenchymal transition in cancer development and its clinical significance.," *Cancer Sci.*, vol. 101, no. 2, pp. 293–9, Feb. 2010.

[29] M. H. Chang, K. Lee, K.-Y. Lee, Y. S. Kim, Y. K. Kim, and J.-H. Kang, "Prognostic role of integrin β1, E-cadherin, and rac1 expression in small cell lung cancer.," *APMIS*, vol. 120, no. 1, pp. 28–38, Jan. 2012.

[30] Y. Liu, N. Zhang, Y. Wang, M. Xu, N. Liu, X. Pang, J. Cao, N. Ma, H. Pang, L. Liu, and H. Zhang, "Zinc finger E-box binding homeobox 1 promotes invasion and bone metastasis of small cell lung cancer in vitro and in vivo.," *Cancer Sci.*, vol. 103, no. 8, pp. 1420–8, Aug. 2012.

[31] E. Arriola, I. Cañadas, M. Arumí-Uría, M. Dómine, J. A. Lopez-Vilariño, O. Arpí, M. Salido, S. Menéndez, E. Grande, F. R. Hirsch, S. Serrano, B. Bellosillo, F. Rojo, A. Rovira, and J. Albanell, "MET phosphorylation predicts poor outcome in small cell lung carcinoma and its inhibition blocks HGF-induced effects in MET mutant cell lines.," *Br. J. Cancer*, vol. 105, no. 6, pp. 814–23, Sep. 2011.

[32] A. J. Gilchrist, R. Meuser, J. Turchinsky, A. R. E. Shaw, M. Pasdar, and W. T. Dixon, "Cell adhesion-mediated transformation of a human SCLC cell line is associated with the development of a normal phenotype.," *Exp. Cell Res.*, vol. 276, no. 1, pp. 63–78, May 2002.

[33] A. F. Gazdar, P. A. Bunn, J. D. Minna, and S. B. Baylin, “Origin of human small cell lung cancer.,” *Science*, vol. 229, no. 4714, pp. 679–80, Aug. 1985.

[34] R. Stovold, S. L. Meredith, J. L. Bryant, M. Babur, K. J. Williams, E. J. Dean, C. Dive, F. H. Blackhall, and A. White, “Neuroendocrine and epithelial phenotypes in small-cell lung cancer: implications for metastasis and survival in patients.,” *Br. J. Cancer*, vol. 108, no. 8, pp. 1704–11, May 2013.

[35] M. Hosokawa, H. Kenmotsu, Y. Koh, T. Yoshino, T. Yoshikawa, T. Naito, T. Takahashi, H. Murakami, Y. Nakamura, A. Tsuya, T. Shukuya, A. Ono, H. Akamatsu, R. Watanabe, S. Ono, K. Mori, H. Kanbara, K. Yamaguchi, T. Tanaka, T. Matsunaga, and N. Yamamoto, “Size-Based Isolation of Circulating Tumor Cells in Lung Cancer Patients Using a Microcavity Array System,” *PLoS One*, vol. 8, no. 6, 2013.

[36] J. Calbo, E. van Montfort, N. Proost, E. van Drunen, H. B. Beverloo, R. Meuwissen, and A. Berns, “A functional role for tumor cell heterogeneity in a mouse model of small cell lung cancer.,” *Cancer Cell*, vol. 19, no. 2, pp. 244–56, Feb. 2011.

[37] T. Tsuji, S. Ibaragi, K. Shima, M. G. Hu, M. Katsurano, A. Sasaki, and G. Hu, “Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth.,” *Cancer Res.*, vol. 68, no. 24, pp. 10377–86, Dec. 2008.

[38] C. L. Hodgkinson, C. J. Morrow, Y. Li, R. L. Metcalf, D. G. Rothwell, F. Trapani, R. Polanski, D. J. Burt, K. L. Simpson, K. Morris, S. D. Pepper, D. Nonaka, A. Greystoke, P. Kelly, B. Bola, M. G. Krebs, J. Antonello, M. Ayub, S. Faulkner, L. Priest, L. Carter, C. Tate, C. J. Miller, F. Blackhall, G. Brady, and C. Dive, “Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer.,” *Nat. Med.*, vol. 20, no. 8, pp. 897–903, Jun. 2014.

[39] T. Yokobori, H. Iinuma, T. Shimamura, S. Imoto, K. Sugimachi, H. Ishii, M. Iwatsuki, D. Ota, M. Ohkuma, T. Iwaya, N. Nishida, R. Kogo, T. Sudo, F. Tanaka, K. Shibata, H. Toh, T. Sato, G. F. Barnard, T. Fukagawa, S. Yamamoto, H. Nakanishi, S. Sasaki, S. Miyano, T. Watanabe, H. Kuwano, K. Mimori, K. Pantel, and M. Mori, “Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis.,” *Cancer Res.*, vol. 73, no. 7, pp. 2059–69, Apr. 2013.

[40] L. Zhang, L. D. Ridgway, M. D. Wetzel, J. Ngo, W. Yin, D. Kumar, J. C. Goodman, M. D. Groves, and D. Marchetti, “The identification and characterization of breast cancer CTCs competent for brain metastasis.,” *Sci. Transl. Med.*, vol. 5, no. 180, p. 180ra48, Apr. 2013.

[41] W. Shen, H. Pang, J. Liu, J. Zhou, F. Zhang, L. Liu, N. Ma, N. Zhang, H. Zhang, and L. Liu, “Epithelial-mesenchymal transition contributes to docetaxel resistance in human non-small cell lung cancer.,” *Oncol. Res.*, vol. 22, no. 1, pp. 47–55, Jan. 2014.

[42] J. K. Rho, Y. J. Choi, J. K. Lee, B.-Y. Ryoo, I. Il Na, S. H. Yang, C. H. Kim, and J. C. Lee, “Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the

sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line.," *Lung Cancer*, vol. 63, no. 2, pp. 219–26, Feb. 2009.

[43] M. Y. Maitah, S. Ali, A. Ahmad, S. Gadgeel, and F. H. Sarkar, "Up-regulation of sonic hedgehog contributes to TGF-β1-induced epithelial to mesenchymal transition in NSCLC cells.," *PLoS One*, vol. 6, no. 1, p. e16068, Jan. 2011.

[44] S. Nakata, K. Sugio, H. Uramoto, T. Oyama, T. Hanagiri, M. Morita, and K. Yasumoto, "The methylation status and protein expression of CDH1, p16(INK4A), and fragile histidine triad in nonsmall cell lung carcinoma: epigenetic silencing, clinical features, and prognostic significance.," *Cancer*, vol. 106, no. 10, pp. 2190–9, May 2006.

[45] S. Al-Saad, K. Al-Shibli, T. Donnem, M. Persson, R. M. Bremnes, and L.-T. Busund, "The prognostic impact of NF-kappaB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer.," *Br. J. Cancer*, vol. 99, no. 9, pp. 1476–83, Nov. 2008.

[46] V. Tirino, R. Camerlingo, K. Bifulco, E. Irollo, R. Montella, F. Paino, G. Sessa, M. V Carriero, N. Normanno, G. Rocco, and G. Pirozzi, "TGF-β1 exposure induces epithelial to mesenchymal transition both in CSCs and non-CSCs of the A549 cell line, leading to an increase of migration ability in the CD133+ A549 cell fraction.," *Cell Death Dis.*, vol. 4, p. e620, Jan. 2013.

[47] A. Bonnomet, L. Syne, A. Brysse, E. Feyereisen, E. W. Thompson, A. Noël, J.-M. Foidart, P. Birembaut, M. Polette, and C. Gilles, "A dynamic in vivo model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer.," *Oncogene*, vol. 31, no. 33, pp. 3741–53, Aug. 2012.

[48] F. M. Davis, T. A. Stewart, E. W. Thompson, and G. R. Monteith, "Targeting EMT in cancer: opportunities for pharmacological intervention.," *Trends Pharmacol. Sci.*, vol. 35, no. 9, pp. 479–88, Sep. 2014.

[49] Y. Yan, Z. Ai, J. Wang, Y. Xu, and Y. Teng, "Influence of epidermal growth factor receptor inhibitor AG1478 on epithelial-mesenchymal transition in endometrial carcinoma cells.," *Int. J. Gynecol. Cancer*, vol. 22, no. 9, pp. 1457–62, Nov. 2012.

[50] S. K. Halder, R. D. Beauchamp, and P. K. Datta, "A specific inhibitor of TGF-beta receptor kinase, SB-431542, as a potent antitumor agent for human cancers.," *Neoplasia*, vol. 7, no. 5, pp. 509–21, May 2005.

[51] J. Schust, B. Sperl, A. Hollis, T. U. Mayer, and T. Berg, "Stattic: a small-molecule inhibitor of STAT3 activation and dimerization.," *Chem. Biol.*, vol. 13, no. 11, pp. 1235–42, Nov. 2006.

[52] K. Siddiquee, S. Zhang, W. C. Guida, M. A. Blaskovich, B. Greedy, H. R. Lawrence, M. L. R. Yip, R. Jove, M. M. McLaughlin, N. J. Lawrence, S. M. Sebti, and J. Turkson, "Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 18, pp. 7391–6, May 2007.

[53] J. T. Thaiparambil, L. Bender, T. Ganesh, E. Kline, P. Patel, Y. Liu, M. Tighiouart, P. M. Vertino, R. D. Harvey, A. Garcia, and A. I. Marcus, "Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation.," *Int. J. Cancer*, vol. 129, no. 11, pp. 2744–55, Dec. 2011.

[54] G. Lahat, Q.-S. Zhu, K.-L. Huang, S. Wang, S. Bolshakov, J. Liu, K. Torres, R. R. Langley, A. J. Lazar, M. C. Hung, and D. Lev, "Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies.," *PLoS One*, vol. 5, no. 4, p. e10105, Jan. 2010.

[55] H. Tanaka, E. Kono, C. P. Tran, H. Miyazaki, J. Yamashiro, T. Shimomura, L. Fazli, R. Wada, J. Huang, R. L. Vessella, J. An, S. Horvath, M. Gleave, M. B. Rettig, Z. A. Wainberg, and R. E. Reiter, "Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance.," *Nat. Med.*, vol. 16, no. 12, pp. 1414–20, Dec. 2010.

[56] C. L. Chaffer, J. P. Brennan, J. L. Slavin, T. Blick, E. W. Thompson, and E. D. Williams, "Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2.," *Cancer Res.*, vol. 66, no. 23, pp. 11271–8, Dec. 2006.

[57] J. A. Sparano, P. Bernardo, P. Stephenson, W. J. Gradishar, J. N. Ingle, S. Zucker, and N. E. Davidson, "Randomized phase III trial of marimastat versus placebo in patients with metastatic breast cancer who have responding or stable disease after first-line chemotherapy: Eastern Cooperative Oncology Group trial E2196.," *J. Clin. Oncol.*, vol. 22, no. 23, pp. 4683–90, Dec. 2004.

[58] S.-D. Huang, Y. Yuan, X.-H. Liu, D.-J. Gong, C.-G. Bai, F. Wang, J.-H. Luo, and Z.-Y. Xu, "Self-renewal and chemotherapy resistance of p75NTR positive cells in esophageal squamous cell carcinomas.," *BMC Cancer*, vol. 9, p. 9, Jan. 2009.

[59] K. H. Tang, Y. D. Dai, M. Tong, Y. P. Chan, P. S. Kwan, L. Fu, Y. R. Qin, S. W. Tsao, H. L. Lung, M. L. Lung, D. K. Tong, S. Law, K. W. Chan, S. Ma, and X. Y. Guan, "A CD90(+) tumor-initiating cell population with an aggressive signature and metastatic capacity in esophageal cancer.," *Cancer Res.*, vol. 73, no. 7, pp. 2322–32, Apr. 2013.

[60] J. K. Smit, H. Faber, M. Niemantsverdriet, M. Baanstra, J. Bussink, H. Hollema, R. P. van Os, J. T. M. Plukker, and R. P. Coppes, "Prediction of response to radiotherapy in the treatment of esophageal cancer using stem cell markers.," *Radiother. Oncol.*, vol. 107, no. 3, pp. 434–41, Jun. 2013.

[61] B. A. Grotenhuis, W. N. M. Dinjens, B. P. L. Wijnhoven, P. Sonneveld, A. Sacchetti, P. F. Franken, H. van Dekken, H. W. Tilanus, J. J. B. van Lanschot, and R. Fodde, "Barrett's oesophageal adenocarcinoma encompasses tumour-initiating cells that do not express common cancer stem cell markers.," *J. Pathol.*, vol. 221, no. 4, pp. 379–89, Aug. 2010.

[62] Y. Zhao, Q. Bao, B. Schwarz, L. Zhao, J. Mysliwietz, J. Ellwart, A. Renner, H. Hirner, H. Niess, P. Camaj, M. Angele, S. Gros, J. Izbicki, K.-W. Jauch, P. J. Nelson, and C. J. Bruns, "Stem cell-like side populations in esophageal cancer: a source of chemotherapy resistance and metastases.," *Stem Cells Dev.*, vol. 23, no. 2, pp. 180–92, Jan. 2014.

[63] M. Quante, G. Bhagat, J. A. Abrams, F. Marache, P. Good, M. D. Lee, Y. Lee, R. Friedman, S. Asfaha, Z. Dubeykovskaya, U. Mahmood, J.-L. Figueiredo, J. Kitajewski, C. Shawber, C. J. Lightdale, A. K. Rustgi, and T. C. Wang, "Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia.," *Cancer Cell*, vol. 21, no. 1, pp. 36–51, Jan. 2012.